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Research article

Herbivory responsive C2H2 zinc finger transcription factor protein StZFP2 from potato

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ABSTRACT

While C2H2 zinc finger transcription factors (TF) are often regulated by abiotic stress, their role during insect infestation has been overlooked. This study demonstrates that the transcripts of the zinc finger transcription factors *StZFP1* and *StZFP2* are induced in potato (*Solanum tuberosum* L.) upon infestation by either the generalist tobacco hornworm (THW, *Manduca sexta* L.) or the specialist Colorado potato beetle (CPB, *Leptinotarsa decemlineata* Say). StZFP1 has been previously characterized as conferring salt tolerance to transgenic tobacco and its transcript is induced by *Phytophthora infestans* and several abiotic stresses. StZFP2 has not been characterized previously, but contains the hallmarks of a C2H2 zinc finger TF, with two conserved zinc finger domains and DLN motif, which encodes a transcriptional repressor domain. Expression studies demonstrate that *StZFP2* transcript is also induced by tobacco hornworm and Colorado potato beetle. These observations expand the role of the C2H2 transcription factor in potato to include the response to chewing insect pests.

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1. Introduction

Plants have evolved an impressive array of responses to environmental stressors such as saline soils, temperature extremes, insect pests, and pathogens. Whether abiotic or biotic, stressors are acutely sensed by the plant, rapidly triggering hormonally mediated reactions. While abscisic acid (ABA) is often associated with salt, cold and drought stress, salicylic acid (SA) with biotrophic pathogens and sucking insect attack, and jasmonic acid (JA) with chewing insect or necrotrophic pathogen attack, this is an oversimplification of the association between stressor and response. For example, SA, JA and ABA may all be utilized as signaling molecules upon attack by chewing insects. Ethylene, brassinosteroids, cytokinins, auxins and gibberellins may also be involved (Erb et al., 2012; Pieterse et al., 2012). These factors, alone or in combination, result in the induction or repression of numerous genes in response to both the physical damage inflicted by the insect, and by specific elicitors that may be present in the insect's regurgitant or saliva (Wu and Baldwin, 2010; Kim et al., 2011). Microarray studies detailing the genes affected have been performed in numerous plant species including potato (Reymond et al., 2004; De Vos et al., 2005; Halitschke et al., 2001; Lawrence et al., 2008). Genes have been identified that play either a direct or indirect role in defense; direct defenses can involve enzymes producing insecticidal toxins or feeding deterrents such as proteinase inhibitors, while indirect defenses involve the production of volatiles that attract natural enemies of the herbivore to the plant. Genes of proteins that may play a regulatory role in defense against insects have also been identified by the same microarray studies, and their roles in transcriptional responses of plants to stressors are now being enumerated (Wu and Baldwin, 2010; Kazan and Manners, 2012). These genes encode numerous hormone receptors, transcriptional activators, repressors and additional proteins controlling gene expression to fine-tune plant responses to environmental challenges.

The Q-type C2H2 zinc finger transcription factor (TF) represents one such factor identified in plant responses to environmental stress. These proteins serve critical roles in many stress response pathways, often leading to regulation of downstream genes

Abbreviations: ABA, abscisic acid; CPB, Colorado potato beetle; JA, jasmonic acid; OS, oral secretions; SA, salicylic acid; TF, transcription factor; THW, Tobacco hornworm.

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required for tolerance to these challenges. The name C2H2 refers to the zinc finger domain, a stretch of 30 amino acids that includes two Cys and two His residues bound by a zinc ion. First discovered in petunia by Takatsuji et al. (1992), a total of 21 have been described in petunia (Kubo et al., 1998). The proteins fall into three groups based on the number of zinc finger domains, most of which contain invariant QALGGH motifs, sometimes referred to as Q-type. Each amino acid of this motif is critical for DNA binding activity; a hydrophobic region with a conserved DLN motif was common to all of the petunia proteins. While using *in silico* methods Englbrecht et al. (2004) described 176 C2H2 zinc finger proteins in Arabidopsis divided into 3 groups, A, B and C. The C1 family contains many proteins responsive to environmental stress (Ciftci-Yilmaz and Mittler, 2008; Kielbowicz-Matuk, 2012). The conserved DLN domain of the C1 family is similar to the first active repression motif described in plants (Ohta et al., 2001; Sakamoto et al., 2004), which was named an ethylene-responsive element-binding factor (ERF)-associated amphiphilic repression or EAR domain. Three amino acid changes in this domain of the Arabidopsis protein ZAT7 resulted in plants with lower salinity tolerance (Ciftci-Yilmaz et al., 2007). Several C2H2 TFs responsive to stress have been characterized in the Solanaceae. Previously only one C2H2 zinc finger containing TF has been characterized in *Solanum tuberosum* StZFP1, which enhances salt tolerance when ectopically expressed in tobacco (Tian et al., 2010). Another C2H2 TF has been characterized from *Capsicum annum*, CaZFP1, that enhances tolerance to drought and infection by *Pseudomonas syringae* pv. tomato when expressed in Arabidopsis (Kim et al., 2004). Two C2H2 TFs have been identified in tobacco NtZFT1 and NTZFP1 are both responsive to spermine (Mitsuya et al., 2007). Lastly, the tomato SlCZFP1, enhances cold tolerance in Arabidopsis and rice (Zhang et al., 2011).

Although many of the Q-type C2H2 TFs have been implicated in salt and drought tolerance, their role in herbivory is less well documented. The current study introduces two C2H2 zinc finger transcription factors induced by herbivory in potato by both the specialist Colorado potato beetle (CPB) and the generalist tobacco hornworm (THW). Gene profiling of CPB infestation of potato revealed the induction of two C2H2 TFs STMEV47 and STMFA43 (Lawrence et al., 2008). This study focuses on STMEV47, renamed StZFP2 for clarity, that responds strongly to herbivory, and also implicates a place in the herbivory response for the previously described "abiotic" finger StZFP1 (Tian et al., 2010), which is 96% identical to STMFA43. The StZFP2 cDNA was cloned and the predicted protein sequence analyzed for the hallmarks of a C2H2 transcription factor. Expression of both *StZFP2* and *StZFP1* transcript was examined under biotic stress and the stress hormones JA, SA and ABA. The cDNA of StZFP2 was also fused to EGFP and used to transiently transform onion epidermal cells to confirm nuclear localization of the fusion protein.

2. Materials and methods

2.1. Insect rearing and regurgitant isolation

CPB were supplied by the USDA-ARS Invasive Insect Biocontrol and Behavior Laboratory. Eggs originated from the New Jersey Department of Agriculture in 1996. Field-collected insects from potato fields at the USDA—ARS Beltsville Agricultural Research Center (Beltsville, MD, USA) were introduced annually to maintain genetic diversity. CPB were reared on *S. tuberosum* L. var. Kennebec and maintained in a laboratory under a 16:8 (L to D) cycle at approximately 25 °C. CPB eggs from the colony were hatched on potato leaves and reared to 3rd instar for infestation.

THW larvae were also supplied by the USDA-ARS Invasive Insect Biocontrol and Behavior Laboratory and were maintained with artificial diet. Eggs originated from Carolina Biological Supply (Burlington, NC, USA). THW eggs from the colony were hatched on potato leaves and reared to 3rd instar for infestation.

2.2. Plant materials and treatments

S. tuberosum 'Kennebec' tubers were planted in 4″ pots containing Metro-Mix 360. All plants were maintained in a Conviron ATC26 growth chamber with a 16:8 (light to dark) cycle at 25 °C during the light phase and 20 °C during the dark phase.

2.2.1. Infestation of potato with THW or CPB

Time course experiments were performed in which control non-infested plants were compared to plants infested continuously for the specified time. Plants were sampled at 2 h, 4 h, 7 h, and 23 h. The fifth leaf from the bottom was harvested for control material. For infested material, the entire plant was enclosed in a fine mesh bag along with ten nonstarved CPB larvae or six nonstarved THW larvae. Only infested leaves were harvested at the appropriate time points.

2.2.2. Hormone treatment of potato plants

For experiments testing the effect of hormones, the abaxial surface of the leaves of four week old potato plants were sprayed with either 50 μM JA in 0.02% DMSO, 500 μM SA or 100 μM ABA in 0.1% ethanol. All chemicals were from Sigma (St Louis, MO, USA) Plants were harvested at 0, 1, 4, 7 and 24 h with 4 biological replicates/time point in a growth chamber as described above.

2.3. RNA isolation and qRT-PCR

For isolation of insect infested leaf RNA, RNeasy Plant Mini Kit was used according to the manufacturer's protocol (Qiagen, Valencia, CA, USA), and included an on column DNase digestion step. Leaf RNA for qRT-PCR was isolated using Trizol. Samples were DNase treated and column purification was completed with RNeasy MinElute Cleanup kit (Qiagen, Valencia, CA, USA). Integrity of RNA was determined with an Experion automated electrophoresis system for RNA (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. A *S. tuberosum* 18S (St18S) sequence from Genbank X67238.1 was used as an endogenous control in qRT-PCR studies. The following marker genes were used in expression studies: *StPR1* (AJ250136.1) for SA treatment, *StPIN2* (KJ475532) for late induction by JA and ABA, *StLOX3* (X96406.1) for early induction with infestation and JA treatment. Custom Taqman assays were designed for each gene used for qRT-PCR (Life Technologies, Carlsbad, CA, USA). Sequences for the primer/probe combinations used in this study are listed in Table 1 cDNA was synthesized using 1ug RNA and Superscript III First Strand Synthesis SuperMix for qRT-PCR (Life Technologies, Carlsbad, CA, USA). The qRT-PCR was performed using the 7500 Real Time PCR System and Taqman assays were performed according to manufacturer's guidelines using 25 ng cDNA (Life Technologies, Carlsbad, CA, USA). The Comparative Ct method (Schmittgen and Livak, 2008) was used to calculate transcript abundance for each gene. Data was analyzed using SAS 9.2 statistical analysis software package. The infestation data was analyzed using three factor analysis of variance (ANOVA) and the hormone data was analyzed using ANOVA with the LSD test to separate means.

2.4. Cloning of StZFP2 and constructs for nuclear localization

StZFP2 (BQ121105.2) was cloned using cDNA generated from CPB infested potato RNA using Titanium™ One-Step RT-PCR Kit (Clontech, Mountain View, CA, USA) according to manufacturer's

Table 1
Custom Taqman primers and probes used in expression studies.

Gene name	Annotation	Accession number	Primer location	Forward primer	Reverse primer	FAM-probe
StZFP2	C2H2 TF	BQ121105.2	115–185	AGTAGAGGCCATGGCTAATTGTG	CTGATGATGAAGAAGTGTTTTTGAAACGA	CAACAGCGCCATTAAG
StZFP1	C2H2 TF	EF093186	349–420	GGGCATAAAGCAAGTCACCGTATAA	AGACGTCGACGGATTGTTATCAC	CCTGCCACCGGAGACG
StLOX3	Lipoxygenase 3	X96406	1448–1520	CCAGTAAGCAAGCTTGATCCTGAAA	GACCAAGAATGTGCTCCTCCTT	ATGGCCCTCAAGAATC
StPIN2	Proteinase inhibitor II	KJ475532	203–342	GTTGTGCAGGTTATAAAGGTTGCA	TGAACGGAGACATTTTGAATAGGCAATAT	TTGGGTCAGATTCTCC
StPR1	Pathogenesis related 1	AJ250136	303–387	GGAGAAGCCAAACTACAACTATGGT	GTTGCGCCAGACTACTTGAGTATAG	CCGCACACTTGTCCGC
St18S	Ribosomal RNA subunit 18S	X67238	1010–1112	CGTCCTAGTCTCAACCATAAACGAT	CCCGGAACCCAAAAACTTTGATT	ACATCCGCCGACCCCT

protocol using primers 5′ StZFP2 5′-CAATTTGTATAAATCTTCAATAA TATGACATCTATG-3′ (BQ121105.2 47–82 bp) and 3′ StZFP2 5′-GTAA TTAATCTTGACATAGATATCTGAGTAAG-3′(BQ121105.2 696–727 bp). The resulting cDNA was ligated to pGEM-T according to the manufacturer's protocol (Promega, Madison, WI, USA) and sequenced.

StZFP2 cDNA was amplified with PCR using CTF/NTF.EV47forward 5′-CACCATGACATCTATGAAAAGAAGCAGAGAAGACA-3′ and either and NTF.EV47reverse 5′-AAAAAAGCATCGCAAAACAGGAGA-3′ for the N-terminal fusion or CTF.EV47reverse 5′-AAGGGATTTAAAAA AAGCATCGCAA-3′ for the C-terminal fusion. Both PCR products were individually ligated to the GATEWAY™ entry vector pENTR/D-TOPO (Life Technologies, Carlsbad, CA, USA) to create either pENTR.EV47NTF or pENTR.EV47CTF. Recombination with p2GWF7 (Karimi et al., 2002) was performed using LR Clonase II Enzyme mix (Life Technologies, Carlsbad, CA, USA) according to manufacturer's protocol to create either C-terminal fusion constructs pZFP2-EGFP or N-terminal EGFP fusion construct pEGFP-ZFP2. All constructs were sequenced to confirm the integrity of the fusion protein sequence.

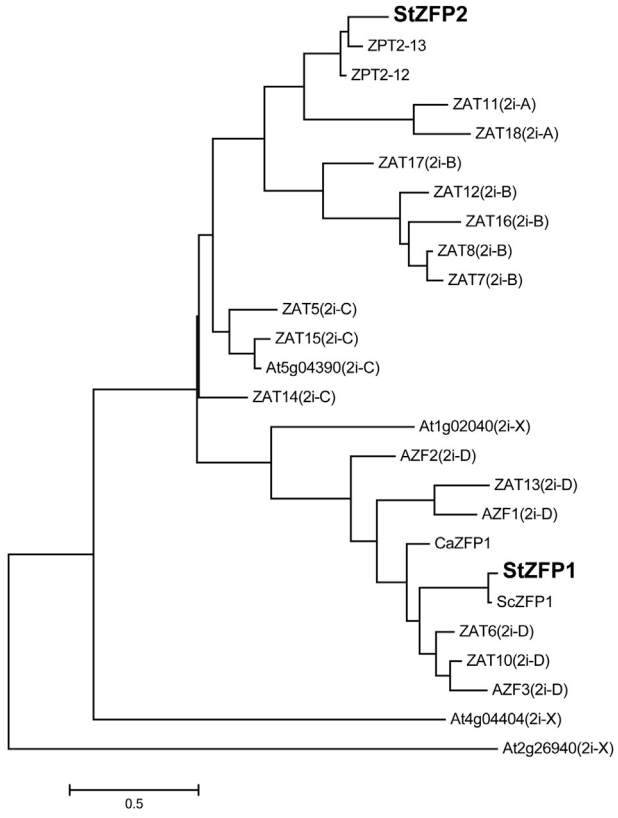

Fig. 1. Arabidopsis Q-type C2H2 TFs containing two zinc fingers align into 4 groups, 2i-A, B, C and D with outlying proteins labeled X. Arabidopsis sequences are labeled with their groups and are from Englbrecht et al. (2004). StZFP2 and the petunia zinc finger proteins ZPT2-12 (BAA21921.1) and ZPT2-13 (BAA21922.1) grouped with Arabidopsis proteins Zat11 and Zat18, of group 2i-A. ScZFP1 (AY704178) from *Solanum chacoense*, StZFP1 (EF093186) from *Solanum tuberosum* and CaZFP1 (AAP41717.1) from *Capsicum annum* grouped with Arabidopsis proteins belonging to 2i-D. Phylogenetic analysis was performed with the Maximum Likelihood Method using MEGA 5.2.

2.5. Nuclear localization

pGWF7 and pZFP2.EGFP were introduced into onion epidermal cells using particle bombardment following the protocol in Weigel and Glazebrook (2002). EGFP expression was visualized using a Nikon Eclipse E600 compound microscope with a Chroma endow GFP band-pass emission filter (HQ470/40 nm EX, Q495LP BS, HQ525/50 nm EM). Images were recorded using a Nikon DXM digital camera.

2.6. Alignment and phylogenetic analysis of StZFP1 and StZFP2

For the phylogenetic tree, proteins were aligned using CLUSTSALW and a phylogenetic tree was inferred using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). The tree with the highest log likelihood (−1320.2934) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 29 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 50 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.2 (Tamura et al., 2011). The protein sequences and their GENBANK number are listed in the caption of Fig. 1. The Arabidopsis proteins are from Englbrecht et al. (2004).

Alignment of StZFP1-like and StZFP2-like proteins was performed using CLUSTALW2 from http://www.ebi.ac.uk/Tools/msa/clustalw2/. The proteins were analyzed using the program MEME (Multiple motifs for EM Elicitation) at (http://meme.nbcr.net/meme/cgi-bin/meme.cgi) to identify conserved motifs. The program cNLS Mapper http://nls-mapper.iab.keio.ac.jp/cgibin/NLSMapper form.cgi was used to identify important importin α dependent nuclear localization signals.

3. Results

3.1. Analysis of StZFP1 and StZFP2 protein sequences

Using Maximum Likelihood, a phylogenetic tree (Fig. 1) was constructed based on the amino acid sequences of StZFP1 and StZFP2, other characterized petunia and Solanaceous C2H2 TF proteins, and the 20 proteins in Arabidopsis containing two zinc finger motifs (Englbrecht et al., 2004). In Arabidopsis these proteins fall into 4 groups, 2i-A, -B, -C and -D with outlying proteins labeled

StZFP1-like

```
                                                    B-box              L-
ZAT10          MALEALTSPRLAS-PIPPLFEDSSV-FHGVEHWTKGK-RSKRSRSDFHH-QNLTEEEYLA 56
ZAT6           MALETLTSPRLSS-PMPTLFQDSALGFHG----SKGK-RSKRSRSEFDR-QSLTEDEYIA 53
AZF3           MALEALNSPRLV---------EDPLRFNGVEQWTKCKKRSKRSRSDLHHNHRLTEEEYLA 51
CaZFP1         MALEALNSPTGTPTPPPFQFESDGQQLRYIENWRKGK-RSKRSRSMEHQ---PTEEEYLA 56
ScZFP1         MPIEALKSPT-AALPPLEEIDDSHN----LDSWAKGK-RSKRPRIDNPP----TRDQYLA 50
StZFP1         MSIEALKSPTAAALPPLEEIDDSHN----LDSWAKGK-RSKRPRIENPP----TPDQYLA 51

                Box
ZAT10          FCLMLLARD------------------NR---------QPPPPPAVEKLSYKCSVCDKTF 89
ZAT6           LCLMLLARDGD----------------RNRDLDLPSSSSSPPLLPPLPTPIYKCSVCDKAF 98
AZF3           FCLMLLARDG---------------------------GDLDSVTVAEKPSYKCGVCYKTF 84
CaZFP1         LCLIMLARSGGS-----VNHQRSLPPPAPVMKLHAPSSSSAAEEEKEKMVYKCSVCGKGF 111
ScZFP1         LCLLMLANDDGTGFGKGKGKGTGSIDVVIEQQQEKK-LKPVFIKEKTEQLFKCSECPKVF 109
StZFP1         LCLLMLANDDGTGFGKGK--GTGSIGVVIEQQQEKKLLKPVFIKEKTEQLFKCSECPKVF 109

                 zinc finger
ZAT10          SSYQALGGHKASHRKNLSQTLSGGGDDHSTSSATTTSA-----VTTGSG---KSHVCTIC 141
ZAT6           SSYQALGGHKASHRKSFSLTQSAGGDELSTSSAITTSG-----ISGGGGGSVKSHVCSIC 153
AZF3           SSYQALGGHKASHR-----SLYGGGENDKSTPSTAV----------------KSHVCSVC 123
CaZFP1         GSYQALGGHKASHR-----KLVPGGDDQSTTSTTTNATGTTTSVNGNGNRSGRTHECSIC 166
ScZFP1         TSYQALGGHKASHR-KINVTATGDDDNNPSTSTSTSGG----VNISALNPSGRSHVCSIC 164
StZFP1         TSYQALGGHKASHR-IINVPATGDGDNNPSTSTSTSG------NISALNPSGRSHVCSVC 162

                       zinc finger
ZAT10          NKSFPSGQALGGHKRCHYEGNNN---------------INTSSVSNSEGAGSTSHVSSS- 185
ZAT6           HKSFATGQALGGHKRCHYEGKNGG--------------GVSSSVSNSEDVGSTSHVSSG- 198
AZF3           GKSFATGQALGGHKRCHYDG----------------------GVSNSEGVGSTSHVSSSS 161
CaZFP1         HKCFPTGQALGGHKRCHYDGGIGNG-------------NANSGVSASVGVTSSEGVGSTV 213
ScZFP1         QKAFPTGQALGGHKRRHYEGKLGGNNRYISGGGCGEGVHSGSVVTTSDGGSGNGGAASTP 224
StZFP1         QKAFPTGQALGGHKRRHYEGKLGGNNRYISGGGCGEGLHSGSVVTTSDGG-----GASTP 217

                   EAR-motif
ZAT10          -HRGFDLNIPPIPEFSM-----------VNGDDEVMSPMPAKKPRFDFPVKLQL--- 227
ZAT6           -HRGFDLNIPPIPEFSM-----------VNGDEEVMSPMPAKKLRFDFPEKP----- 238
AZF3           -HRGFDLNIIPVQGFS--------------PDDEVMSPMATKKPRLK---------- 193
CaZFP1         SHRDFDLNIPALPEFWLGF---------GSGEDEVESPHPAKKSRLCLPPKYELFQH 261
ScZFP1         IARDFDLNMPALPGWQLDLTIDCGGRSQFPIEQEVESPMPAKKPRLFFD-------- 273
StZFP1         VARDFDLNMPASPGCQLDLTIDCGGRSQHPIEQEVESRMPAKKPRLFFD-------- 266
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StZFP2-like

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                                  L-box                              zinc
ZPT2-13        MSSIKRSRSEEYGQVEAEAMANC--LMLLSK---LNDHNTSK----NQDHHNEFECKTCN 51
ZPT2-12        MSAMKRSR--EDRQVEAAAMANC--LMLLSK---LNDKSTSTT-TTNQDHHNDFECKTCN 52
StZFP2         MTSMKRSR-EDNMQIEVEAMANCALMALLSR---FKNTSSSS----DHHEINDFECKTCN 52
ZAT11          ---MKRERSDFEESLKNIDIAKC--LMILAQTSMVKQIGLNQHTESHT--SNQFECKTCN 53
ZAT18          ---MKRDRSDYEESMKHIDIVES--LMMLSRSFVVKQIDVKQSTGSKTNHNNHFECKTCN 55

                  finger    B-box                                  zinc finger
ZPT2-13        KRFPSFQALGGHRASHKRTKVLTGAGEFLAQQA--KKNKMHECSICGMEFSLGQALGGHM 109
ZPT2-12        KRFSSFQALGGHRASHKRPKLLIGAGEFLVQPS--SK-KMHECSICGMEFSLGQALGGHM 109
StZFP2         KRFPSFQALGGHRASHNKRSRLLG--DFLVQTN--KKNKMHKCSICGVEFSLGQALGGHM 108
ZAT11          KRFSSFQALGGHRASHKKPKLTVEQKDVKHLSNDYKGNHFHKCSICSQSFGTGQALGGHM 113
ZAT18          RKFDSFQALGGHRASHKKPKLIVDQEQVKHRN---KENDMHKCTICDQMFGTGQALGGHM 112

                                                 EAR-motif
ZPT2-13        RRHRDENNKTLKVARKTTTMIPVLKKSNSSKRIFCLDLNLTPRNEDVDLKLWPTAPISSP 169
ZPT2-12        RRHRAAIDEKSKAATK-AMMIPVLKKSNSSKRIFCLDLNLTPRNEDVDLKLWPTAPISSP 168
StZFP2         RRHRDEINK----ITDEKTMIPILKKSNSSKRIFCLDLNLTPRDDNVDFKLWPTTPIASP 164
ZAT11          RRHRSSMTVEPSFISPMIPSMPVLKRCGSSKRILSLDLNLTPLENDLEY-IFGKTFVPKI 172
ZAT18          RKHRTSMITEQSIVPSVVYSRPVFNRCSSSKEIL--DLNLTPLENDLVL-IFGKNLVPQI 169


ZPT2-13        VLRIFI 175
ZPT2-12        VLRIFI 174
StZFP2         VLRCFF 170
ZAT11          DMKFVL 178
ZAT18          DLKFVN 175
```

Fig. 2. StZFP1-like and StZFP2-like proteins were aligned using CLUSTALW2. Zinc finger motifs (red) with invariant QALGGH, B-box (yellow), L-box (blue), and an EAR-motif (purple) are indicated.

-X. Clearly the Arabidopsis proteins fall into 4 discrete clusters with 2i-A and 2i-B more related than to the other Arabidopsis proteins. StZFP1, ScZFP1, and CaZFP1 formed a cluster with and Arabidopsis proteins from subclass 2i-D, while StZFP2, the petunia proteins ZPT2-12 and ZPT2-13 formed a group with the Arabidopsis subclass 2i-A proteins.

Alignments of the StZFP1-like and StZFP2-like proteins are shown in Fig. 2. While both StZFP1 and StZFP2 contain all the hallmarks of C2H2 zinc finger transcription factors; B-box (nuclear localization signal-NLS), L-Box, EAR motif and 2 zinc finger domains with the conserved QALGGH motif, there are several differences. StZFP1 is 266 amino acids while StZFP2 is considerably smaller at only 170. While the B-box of StZFP1 is predicted to be at the N-terminal end of the protein (although several additional NLS sequences were also identified), it most likely resides between the zinc fingers in StZFP2. The L-box is also closer to the N-terminal end of StZFP2 than it is in StZFP1, and the EAR motif of StZFP2 is of the LXLXL-type while StZFP1 is the DLN-type. Finally, the space between the zinc fingers referred to as the linker is smaller in StZFP2 at 22 amino acids, while the StZFP1 linker is 36 amino acids. Spacing of the zinc fingers is thought to be critical for the specificity of DNA binding and may determine the genes that are repressed by the individual C2H2 zinc finger TF (Takatsuji, 1999).

Constructs were designed fusing the protein sequence of StZFP2 with EGFP as either a C terminal or N-terminal fusion as described in the materials and methods. The StZFP2-EGFP or EGFP-StZFP2 or the EGFP alone were transiently transformed into onion epidermal cells using a biolistic particle gun (Weigel and Glazebrook, 2002). The StZFP2-EGFP produced nuclear-localized EGFP expression while the construct containing EGFP alone resulted in diffuse expression of EGFP throughout the cell (Fig. 3). The EGFP-StZFP2 also resulted in nuclear localization but with slightly less transformation efficiency (data not shown).

3.2. Expression of StZFP1 and StZFP2 transcript in potato

Potato plants were treated with either 500 μM SA, 100 μM ABA, or 50 μM JA as described in the materials and methods. The data were statistically analyzed using ANOVA and significant results were followed by a least significant difference (LSD) test to determine differences between time points (Table 1A and Fig. 4). Marker genes expected to be induced by each hormone were used to confirm a successful hormone response: *StPR1* (pathogenesis response −1) was used as a marker for induction by SA (Navarre and Mayo, 2004). *StPIN2* (proteinase inhibitor II) was used as a marker gene for ABA (Pena-Cortes et al., 1995), and *StLOX3* and *StPIN2* for JA (Pena-Cortes et al., 1995; Royo et al., 1996). *StLOX3*

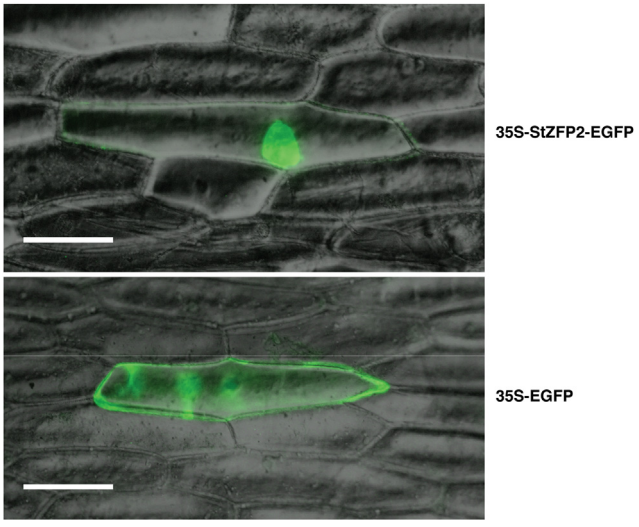

Fig. 3. StZFP2 localized to the nucleus. The fluorescent marker protein EGFP was found in the nucleus when fused with StZFP2 and expressed transiently in onion epidermal cells. Conversely, EGFP alone was expressed throughout the cytoplasm. White size bar = 100 μm.

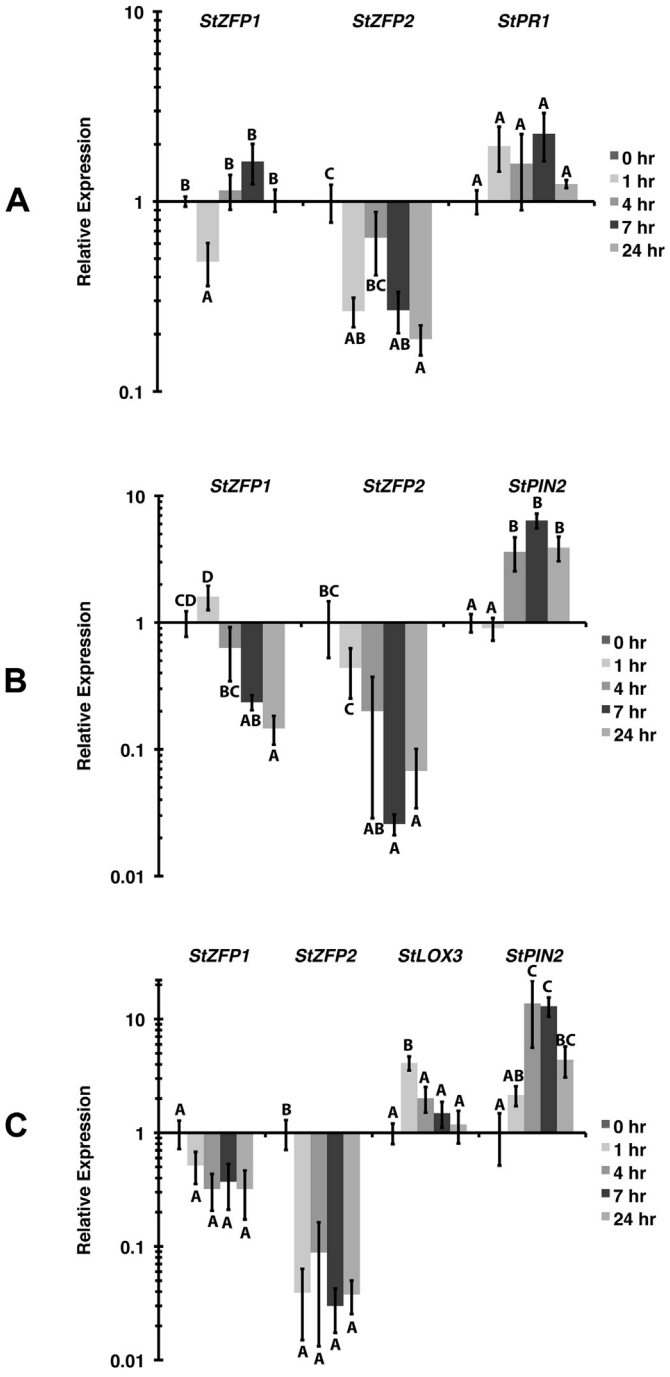

Fig. 4. The effect of (A) salicylic acid, (B) abscisic acid and (C) jasmonic acid on expression of *StZFP1* and *StZFP2*. The relative transcript level for each gene was determined using *St18S* as the endogenous control and the Comparative Ct method of Schmittgen and Livak (2008). Error bars represent standard error of four biological replicates per time point. The data was analyzed using ANOVA with an LSD test for each gene separately and results presented in Table 2A. Within each gene–hormone combination, bars sharing the same letters were not significantly different.

encodes a lipoxygenase (13-LOX) involved in the first step in JA biosynthesis; adding an oxygen at C-13 of linolenic acid. Thus, it mediates plant responses that deter further insect attack. Interference with JA biosynthesis using antisense of *StLOX3* abolished expression of wound-induced proteinase inhibitors such as StPIN2 and resulted in greater weight gain among insects feeding on potato (Royo et al., 1999). Protease inhibitors like PIN2 play a role in defense, as they can deactivate gut proteases of the insect necessary for digestion of nutrients (Chen, 2008).

For each hormone treatment, all marker genes were induced as expected from the prior studies, indicating a successful application. However, while *StPR1* appeared modestly induced by SA, the biological replicates varied widely and failed to be significant by ANOVA (Table 2A and Fig 4A). In potato, basal levels of SA are reportedly much higher than what is found in Arabidopsis or tobacco and also vary depending on potato variety and environmental conditions (Navarre and Mayo, 2004). SA significantly repressed *StZFP1* at 1 h, but the effect was transient and none of the subsequent time points were significantly different from the control. In contrast, *StZFP2* transcripts were reduced 3.8-fold at 1 h, and were also similarly repressed at 7 h and 24 h. Although ABA appeared to transiently increase *StZFP1* transcripts at 1 h it was not statistically significant, and transcripts decreased steadily thereafter; with 7 h and 24 h significantly repressed by 4.3 and 6.8-fold respectively. *StZFP2* was significantly repressed by ABA up to 38-fold peaking at the 7 h determination and persisted for the duration of the experiment (Fig. 4B). Although it appears that JA represses *StZFP1*, the *p* value of 0.0656 does not meet the significant threshold (Table 2A). JA significantly repressed *StZFP2* ca. 10–30 fold over the entire 24 h test period (Fig. 4C).

Potatoes were infested with THW larvae continuously for 2, 4, 7 and 23 h as described in the materials and methods. *StLOX3* was chosen as a marker gene in the current study for its early induction and link to insect defense (Royo et al., 1996, 1999). In control (non-infested) plants harvested at 0, 2, 4, 7 and 23 h, *StZFP2* expression varied only one half-fold over this time period (data not shown). In THW infested plants, expression of *StLOX3*, and *StZFP1* peaked at 4 h of infestation, *StZFP2* at 7 h, with *StZFP2* induced 80 fold by 4 h and 7 h (Fig. 5A) compared to the 0 h control. Infestation of potato with CPB was also performed and the leaves of control (non-infested) plants were also harvested at 0, 2, 4, 7 and 23 h, while infested leaves were harvested at 2, 4, 7 and 23 h. The level of *StZFP1* and *StZFP2* transcript did not change in the controls over this time (data not shown). Infestation resulted in induction of *StLOX3*, *StZFP1* and *StZFP2*, with the greatest induction occurring at 2 h and decreasing somewhat thereafter (Fig. 5B). A three factor ANOVA performed on the infestation data for each gene separately (Table 2B) revealed that all three were significantly induced by either CPB or THW infestation throughout the entire time period.

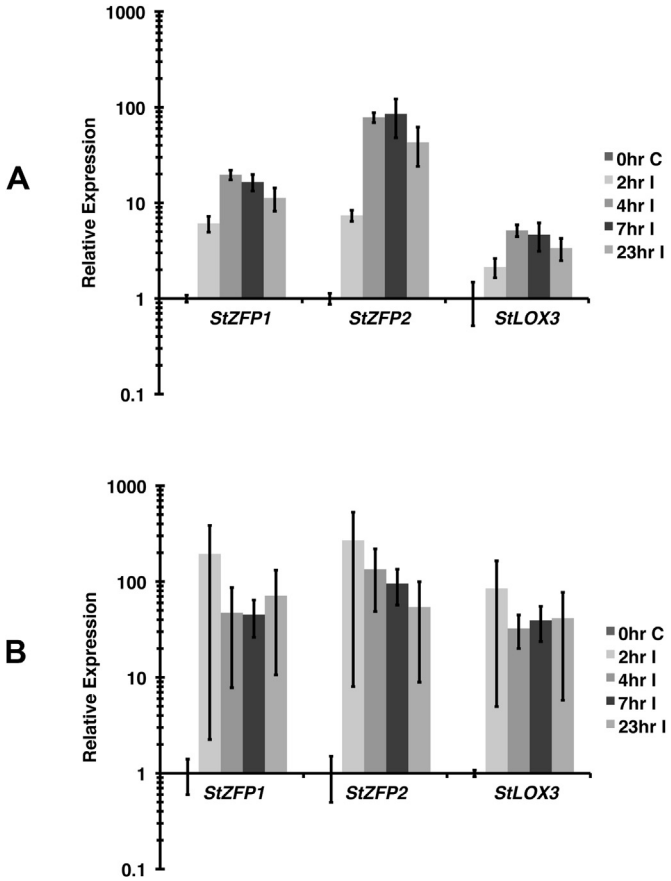

Fig. 5. Infestation of potato by A) THW or B) CPB increased levels of *StZFP1*, *StZFP2*, and *StLOX3* transcript. The relative transcript level for each gene was determined using *St18S* as the endogenous control, and the Comparative Ct method of Schmittgen and Livak (2008). The error bars represent standard error of three biological replicates per time point. Three factor ANOVA was performed and results presented in Table 2B.

4. Discussion

StZFP1 and StZFP2 are highly similar to the Arabidopsis C2H2 TF subfamily C1-2i containing two zinc finger domains. The C1-2i

Table 2A
Statistical analysis of gene expression upon hormone treatment from Fig. 4.

Gene name	*F*	*df*	*p*
SA			
StZFP1	5.92	4, 12	0.0072
StZFP2	9.11	4, 12	0.0013
StPR1	1.08	4, 12	0.4076
ABA			
StZFP1	10.16	3, 12	0.0008
StZFP2	4.094	3, 12	0.0138
StPIN2	10.06	3, 12	0.0008
JA			
StZFP1	2.94	4, 12	0.0656
StZFP2	14.09	4, 11	0.0003
StLOX3	5.59	4, 12	0.0089
StPIN2	7.52	4, 10	0.0046

Table 2B
Statistical analysis of gene expression upon infestation from Fig. 5.

Gene name	*F*	*df*	*p*
STZFP1			
Insect	0.11	1, 31	0.748
Infestation	91.59	1, 31	0.0001
Time	0.22	3, 31	0.8785
Insect x infestation	0.07	1, 31	0.797
STZFP2			
Insect	1.37	1, 32	0.2501
Infestation	134.01	1, 32	0.0001
Time	0.7	3, 32	0.5616
Insect x infestation	0	1, 32	0.9793
STLOX3			
Insect	3.74	1, 31	0.0623
Infestation	107.82	1, 31	0.0001
Time	0.25	3, 31	0.8597
Insect x infestation	0.75	1, 31	0.3936

proteins of Arabidopsis have been divided into four groups (A-D) and ungrouped proteins (X) based on sequence similarity (Fig. 1). Among the Arabidopsis proteins, StZFP2 appears most closely related to the C1-2i-A group, which includes Zat11 and Zat18. StZFP2 appears even more similar to the petunia proteins ZPT2-12 and ZPT2-13. Aside from numerous global expression studies using microarray, no specific characterizations of the Arabidopsis proteins in the C1-2i-A cluster have been performed. Several properties of the StZFP2 protein indicate that it is a Q-type C2H2 TF: The two conserved zinc finger domains outlined in Fig. 2, which are crucial for DNA binding (Kubo et al., 1998), and the EAR domain involved in transcriptional repression of genes (Ohta et al., 2001). In addition, the StZFP2-EGFP fusion protein localized in the nucleus (Fig. 3) as expected of a DNA binding transcription factor.

The larger TF StZFP1 falls into the C1-2i-D group with the Arabidopsis proteins Zat6, Zat10 and AZF3. Other near neighbors of StZFP1 include; CaZFP1 from pepper, ZPT2-3 from petunia, NtZP1 from tobacco, and SlCZFP1 from tomato. In contrast to the C1-2i-A, many of the C1-2i-D proteins have been functionally characterized. The results of these studies have shown these TFs to be induced by abiotic stresses such as temperature extremes, drought or salt, and by hormones associated with induction of stress responses (Kielbowicz-Matuk, 2012; Kim et al., 2004; Zhang et al., 2011; Mittler et al., 2006), a role in responding to a pathogen was also implicated (Kim et al., 2004). Consequently most of these TFs were studied with abiotic stress related hormones such as ABA or when there was pathogen induction of the TF by SA. In the current study assessment of the transcriptional response of *StZFP1* and *StZFP2* to these hormones showed that SA transiently represses *StZFP1* at 1hr (Fig. 4A). *StZFP2* was also repressed 1 hr after application of SA and then again at 7 and 24 h. *StZFP1* was repressed by ABA at 7 and 24 h while *StZFP2* was repressed at 4–24 h (Fig. 4B). Previous reports demonstrate that C1-2i-D TFs appear mixed in their responsiveness to ABA. In Arabidopsis, *AZF2* transcript is strongly induced, while *AZF1* and *AZF3* are not (Sakamoto et al., 2004). In contrast to our results Tian et al. (Tian et al., 2010) found that SA induced *StZFP1* transiently at 2 h while ABA induced *StZFP1* at 12 and 24 h. However, Tian et al. (Tian et al., 2010) used 10 mM SA rather than 500 μM, a different variety of potato, and detached stems. The experiments described here also used realtime qRT-PCR to measure the results while Tian et al. (2010) used semi qRT-PCR, which makes observing repression very difficult.

In the current study, expression of either *StZFP1* or *StZFP2* in potato was induced by infestation by the generalist THW (Fig. 5A) or the specialist CPB (Fig. 5B). The induction of *StZFP2* was rapid (peaking within 2 or 4 h), as one might expect for the response to chewing insect attack. During herbivory, the increase in *StZFP2* transcript dwarfs the responses of the JA induced *StLOX3* marker gene (Fig. 5). Results reported in this work demonstrate that the C1-2i-D protein StZFP1, and the C1-2i-A protein StZFP2, were induced by herbivory. Previously, only Schweizer et al. (2013) has addressed the response of Q-type C2H2 TFs to herbivory. Examining expression of Arabidopsis TFs after feeding by the generalist insect pest *Spodoptera littoralis*, revealed 41 TFs induced by herbivory, including the Q-type C2H2 proteins ZAT10, ZAT12 and AZF2. A role for these TFs in an insect defense response was demonstrated when *S. littoralis* feeding on individual lines of T-DNA mutants for ZAT10, ZAT12 and AZF2 gained more weight than those feeding on the wildtype *Arabidopsis*, Col-0. Interplay between JA, the defense response and the three Q-type C2H2 TFs is suggested by the following: (1) The level of chewing insect induced transcripts for these TFs was lower in the coi1-1 mutant plants, which are dramatically impaired in JA response. (2) AZF2 induction was reduced in the myc234 mutant line which also has a severely impaired JA induced defense response. (3) Finally, in ZAT10 and ZAT12 mutant plants, activation of the necrotrophic pathogen branch of the JA pathway was enhanced for its marker gene, PDF1.2, by *S. littoralis* feeding. Hence, C1-2i-D TFs such as ZAT10, ZAT12 and AZF2 are responsive to both biotic and abiotic stresses (Kielbowicz-Matuk, 2012; Sakamoto et al., 2004; Kim et al., 2004; Zhang et al., 2011; Mittler et al., 2006).

Since StZFP1 and StZFP2 were highly induced by herbivory, which is also known to induce the JA pathway, we suspected that these TFs might be induced by JA itself. However, while the JA marker genes *StLOX3* and *StPIN2* were both induced by JA application, *StZFP2* was significantly repressed (Fig. 4C). Thus, while both *StZFP1* and *StZFP2* expression can be induced along with JA in a plant being attacked by chewing insects, this TF does not appear to be induced by JA itself. At face value, the results suggest that the factor(s) that induce *StZFP1* and *StZFP2* can easily overcome the negative influence JA might exert. Obviously these results should be interpreted with caution; exogenous application of a single hormone probably does not accurately mimic the hormonal signaling and feedback that occurs in an infested plant. Since most C2H2 TFs have been tested for response to abiotic stress, only a subset of these TFs has been tested for their response to JA. Several C1-2i-D-like proteins are responsive to JA in Arabidopsis, pepper, petunia and chickpea (Kielbowicz-Matuk, 2012; Sakamoto et al., 2004; Kim et al., 2004; Pauwels et al., 2008; Jain and Chattopadhyay, 2013), whereas Arabidopsis proteins AZF1 and AZF3 are not induced by JA (Sakamoto et al., 2004). However, C1-2i-B and 2i-C proteins such as PtiZFP1 and PtaZFP2 from poplar are MeJA responsive while PtaZFP1 is not (Hamel et al., 2011; Gourcilleau et al., 2011). Finally an involvement in JA signaling has also been suggested by the binding of AZF2 and ZAT10 to the LOX3 promoter *in vitro* (Pauwels et al., 2008). In aggregate, the literature does not appear to indicate that any single group of Q-type C2H2 TFs is exclusively JA responsive.

Perhaps feeding by THW and CPB involves overcoming JA repression of *StZFP2*. Herbivory can be partially mimicked by mechanical wounding and addition of oral secretions (OS) from the insect pest at the wound site (simulated herbivory). Components of the OS of CPB and THW can alter the plants response. Recent work by Chung et al. (2013) in tomato demonstrates that bacteria found in the OS of CPB down-regulates the JA burst and the JA defense response and enhances the SA burst and SA response. In THW the major component of OS is a fatty acid conjugate, which when applied to wounded *Nicotiana attenuata* mimics the response to herbivory (Halitschke et al., 2001). However not all *N. attenuata* herbivory induced genes are JA responsive. For example, *NaMPK4* is not induced by JA but is responsive to OS from THW during simulated herbivory (Hettenhausen et al., 2013). Consequently the plant response to chewing insects may be influenced by not only the wound response, but components of the OS and microbes associated with the OS.

Given that exogenous application of JA did not significantly repress *StZFP1*, this Q-type C2H2 TF may be mainly associated with control of the response to pathogens such as *Phytophthora infestans* and/or the abiotic response to osmotic/salt demonstrated by over-expression of StZFP1 in tobacco by Tian et al. (Tian et al., 2010). The induction of *StZFP1* by herbivory may simply be secondary to herbivory by chewing insects, which can change osmotic balance due to damage to the vasculature. StZFP2 may be more directly involved in the response to herbivory since it is significantly affected by JA. Plants over-expressing StZFP2, or knocked out of this transcript by RNAi may help to determine its effect on the herbivore.

Most studies on C2H2 TFs in plants have concentrated on abiotic triggers and the role in herbivory has not been well studied. The induction of these TFs upon insect infestation may simply be a

result in changes in osmotic balance upon infection or these TFs may have a broader role than initially envisioned. Since over-expression of STZFP1 in tobacco led to an increase in salt tolerance (Tian et al., 2010), perhaps this transgenic would also be more tolerant to biotic attack. Additionally, the over-expression of StZFP2 may result in a plant more tolerant to salt, or more resistant to insects. These questions are currently under study. However, considering that the strongest response of *StZFP2* transcript is upon herbivory, a direct role of this C2H2 TF in an insect defense response is implicated.

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Contributions

Susan Lawrence conceived the experimental plan. Nicole Novak, Susan Lawrence and Richard Jones performed the experiments. Susan Lawrence performed the phylogenetic and sequence analysis. Nicole Novak, Susan Lawrence and Michael Blackburn analyzed the gene expression data, Robert Farrar performed the statistical analysis. Susan Lawrence, Nicole Novak and Michael Blackburn wrote and edited the manuscript.

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